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AN ALKALINE PROTEASE-SUBTILISIN CARLSBERG BY RECOMBINANT DNA
TECHNIQUES

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(71) Applicant(s)
HENKEL KOMMANDITGESELLSCHAFT AUF AKTIEN

(72) Inventor(s)
Dr. HARALD BERGER; Professor Dr. WERNER GOEBEL; Dr. JURGEN KREFT; Dr.
FRIEDHELM BARTNIK

(74) Attorney or Agent
SPRUSON & FERGUSON, GPO Box 3898, SYDNEY NSW 2001

(56) Prior Art Documents
US 3790482
US 3557002

(57) Claim

1. Isolated, double-stranded deoxyribonucleic acids consisting of
- the following structural gene which only codes for a polypeptide:

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10          20          30          40
...GGGCAAA CGGTCCTTA CGGCATTCCT CTCATTAAG
CGGACAAAGT GCAGCTCAA GGCTTTAAGG GAGCGAATGT
AAAAGTAGCC GTCTGGGATA CAGGAATCCA AGCTTCTCAT
CGGGACTTGA ACGTAGTCGG CGGAGGAAGC TTTGTGGCTL
GCGAAGCTTA TAACACCGAC GGCAACGGAC ACGGCACACA
TGGTGCCGGT ACAGTAGCTG CGCTTGACAA TACAACGGGT
GTATTAGGCG TTGCGCCAAG CGTATCCTTG TACGCGGTTA
AAGTACTGAA TTCAAGCGGA AGCGGATCAT ACAGCGGCAT
TGTAAGCGGA ATCGAGTGGG CGACAACAAA CGGCATGGAT
GTTATCAATA TGAGCCTTGG GGGAGCATCA GGCCTGACAG
CGATGAAACA GGCAGCTGCAC AATGCATATG CAAGAGGGGT
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TGTCGTTGTA GCTGCAGCAG GGAACAGCGG ATCTTCAGGA
AACACGAATA CAATTGGCTA TCCTGCCAAA TACGATTCTG
TCATCGCTGT TGGTGCGGTA GACTCTAACA GCAACAGAGC
TTCATTTTCC AGCCTCGGAG CAGAGCTTGA AGTCATGGCT
CCTGGCGCAG GCGTATACAG CACTTACCCA ACGAACACTT
ATGCAACATT GAACGGAACG TCAATGGCTT CTCCTCATGT
AGCGGGAGCA GCAGCTTTGA TCTTGTCAAA ACATCCGRAC
CTTTCAGCTT CACAAGTCCG CAACCGTCTC TCCAGCACGG
CGACTTATTT GGGAGCTAA TTCTACTATG GGAAGGTCT

stop

GATCAATGTC GAAGCTGCGG CTCAA TAA....

- start and stop codons in an operable sequence,
- a ribosome binding site,
- a promoter,

characterized in that it has the following structure:

- a promoter which is recognized by the RNA-polymerase of microorganisms of the genus *Bacillus*,
- a ribosome binding site,
- a start codon,
- a leader sequence,
- a stop codon,

DNA-sequences containing up to 400 base pairs without any special expression effect optionally being present at both ends.

6. A process for selecting microorganism strains containing hybrid plasmids according to Claims 2 to 5, characterized in that

- in a first selection process, the strains are separated from strains free from hybrid plasmid through the property of resistance acquired by the hybrid plasmid and
- the producers of subtilisin Carlsberg are selected from the resistant strains by coating the plate cultures with a film covered with antibodies against subtilisin Carlsberg and binding the antibody-subtilisin Carlsberg complex to other antibodies against subtilisin Carlsberg which are coupled to a peroxidase, after which a color reaction is carried out with the peroxidase.

2. Hybrid plasmids for microorganisms of the genus *Bacillus*, characterized in that they contain at least one double-stranded DNA according to Claim 1 in operable form.

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FORM 10

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PATENTS ACT 1952

COMPLETE SPECIFICATION

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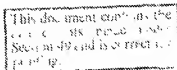
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Name of Applicant: HENKEL KOMMANDITGESELLSCHAFT AUF AKTIEN

Address of Applicant: Henkelstrasse 67, Düsseldorf, Federal Republic of Germany

Actual Inventor(s): HARALD BERGER, WERNER GOEBEL, JÜRGEN KREFT and FRIEDHELM BARTNIK

Address for Service: Spruson & Ferguson, Patent Attorneys, Level 33 St Martins Tower, 31 Market Street, Sydney, New South Wales, 2000, Australia

Complete Specification for the invention entitled:

"AN ALKALINE PROTEASE, A PROCESS FOR THE PREPARATION OF HYBRID VECTORS AND GENETICALLY TRANSFORMED MICROORGANISMS"

The following statement is a full description of this invention, including the best method of performing it known to us

SBR/TGK/182W

D 7143

A B S T R A C T

An alkaline protease, a process for the preparation of hybrid vectors and genetically transformed microorganisms

A structural gene which codes for the protease subtilisin Carlsberg or proteolytically active sub-units or proteolytically active variants thereof, including their leader sequences, is described. Hybrid plasmids and microorganism strains containing this structural gene and also a process for separating these microorganism strains from inactive strains are also described.

Henkelstrasse 67
4000 Dusseldorf, 30.7.1985

HENKEL KGaA
ZR-FE/Patente

Dr. Wi/Hd/Po

Patent Application

D 7143

An alkaline protease, a process for the preparation
of hybrid vectors and genetically transformed microorganisms

1 This invention relates to a method for transforming
microorganisms of the genus *Bacillus* into producers of
the protease subtilisin Carlsberg and variants thereof
and, to that end, describes the preparation of double-stranded
5 deoxyribonucleic acids which only code for subtilisin
Carlsberg or variants thereof, hybrid vectors which contain
these isolated double-stranded deoxyribonucleic acids together
with the ^{structural} ~~structural~~ gene of the leader protein, start and
stop codons in an operable sequence, a ribosome binding
10 site and promotor, and also host organisms of the genus
Bacillus for these hybrid plasmids.

There are several known proteases which are produced
by microorganisms, more especially bacteria and fungi,
and which are exported from the cell by active transport
15 so that they collect in the culture medium. Numerous
enzymes of this type are already being commercially used
in processes where proteins have to be degraded. Important
applications include, for example, detergents and cleaning
preparations and even animal foods. The proteases are
20 normally classified according to the pH-range in which
they show optimum activity and, accordingly, are known
as alkaline, neutral and acidic proteases. The alkaline



- 1 proteases are of particular importance in detergents and
cleaning preparations. One important group is known by
the generic name of subtilisin. This name derives from
Bacillus subtilis because inter alia strains of this species
5 are capable of producing subtilisin. In recent years,
researchers have succeeded in separating from one another
and structurally elucidating various naturally occurring
types of subtilisin (E.L. Smith et al, J. Biol. Chem. (1968)
243 (9), 2184. In this connection, it has been found
10 that two types of subtilisin are of particular importance
in practice, namely subtilisin BPN' and subtilisin Carlsberg.
Subtilisin BPN' is often obtained from Bacillus amylo-
liquefaciens, whereas subtilisin Carlsberg, for example,
may be obtained by cultivation of strains of the species
15 Bacillus licheniformis. Many Bacillus strains often excrete
both proteases in admixture with neutral proteases (metallo-
proteases).

- In view of the commercial significance of enzymes,
which may be regarded as somewhat greater in the case of
20 the protease subtilisin Carlsberg than in the case of
the protease subtilisin BPN', numerous attempts have already
been made to find strains which produce one enzyme or the
other or mixtures thereof and which are also suitable for
commercial preparation of the products. In this connection,
25 reference is made for example to the following German
Offenlegungsschriften or Patent Specifications:
DE 19 40 488, DE 20 18 451, DE 20 44 161, DE 21 01 803,
DE 21 21 397 and DE 29 25 427, and also to GB 1 263 765,
US 3 623 957, US 4 264 738 and EP-A 6 638. The applications
30 or patents mentioned are concerned with classical mutation
processes in which the frequent repetition of mutation
and selection steps ultimately gives product strains which
are optimized in regard to yield and product quality.
It is known among experts that a mutation process such
35 as this takes place statistically so that it is hardly



- 1 likely to give "tailored" strains which have objectively
the highest possible performance level. In addition,
cultivated strains such as these may also tend towards
back-mutations, i.e. they lose at least some of their
5 favorable properties and become "wild".

A significant advance was provided by the gene re-
combination technique (Cohen and Boyer, US 4 237 224).
This technique enables a foreign gene to be expressed and
reproduced in a microorganism by suitably opening a plasmid,
10 connecting it to the foreign double-stranded deoxyribonucleic
acid, reclosing it to form the ring and inserting it
into a suitable host organism. Applying this basic
technique, European Patent Application 0 130 756 describes
the isolation of double-stranded deoxyribonucleic acids
15 which code on the one hand for subtilisin BPN' and on the
other hand for neutral proteases (metallo-proteases).
The insertion of the double-stranded deoxyribonucleic acids
into vectors and the modification thereof is also described.
Although the gene-technological production and modification
20 of hydrolases (i.e. proteases, other amylases, esterases,
etc.) is claimed, it does not seem possible to apply the
steps illustrated with reference to the particular example
of subtilisin BPN' to other systems without inventive
activity. This would appear all the more to be the
25 case since, according to the teaching of the European Patent
Applications cited above, the separation of the gene coding
for hydrolase from the gene pool of the starting organism
is said to take place using a marked oligonucleotide
chain, this oligonucleotide chain corresponding to a
30 partial sequence of the hydrolase and being used in its
various possibilities - as predetermined by the genetic
code. Considerable experimental effort is required of
the expert here, in other words several oligonucleotide
chains have to be synthesized in appropriate purity. In
35 addition, the method involved presupposes knowledge of

the amino acid sequence of the desired product.

The inventors set themselves the task of finding microorganism strains which are capable of producing subtilisin Carlsberg, the generic name subtilisin Carlsberg also being intended to include in particular a variant of the enzyme which has two modifications. Another task was to provide suitable hybrid plasmids which could be inserted into and were stable in microorganisms of the genus *Bacillus* and which code for the production and excretion of subtilisin Carlsberg. Finally, the inventors set out to provide operable, isolated, double-stranded deoxyribonucleic acids which code for subtilisin Carlsberg and which contain the nucleotide sequences necessary for the production and excretion of the enzyme. Another task which the inventors set themselves was to use an immunological detection method simplified by comparison with the isolation method described in European Patent Application 0 130 756 by preformed, marked oligonucleotide sequences in the selection of Carlsberg-positive microorganisms.

In a first embodiment, therefore, the present invention relates to isolated, double-stranded deoxyribonucleic acids consisting of

- a structural gene which only codes for a polypeptide,
- start and stop codons in an operable sequence,
- a ribosome binding site,
- a promoter,

characterized in that they have the following structure;

- promoter recognized by the RNA-polymerase of microorganisms of the genus *Bacillus*,
- ribosome binding site,
- start codon,
- a leader sequence,
- structural gene which codes for subtilisin Carlsberg



1 or its proteolytically active sub-units or proteolytically
active variants, including their leader sequences,
- stop codon,

5 DNA-sequences containing up to 400 base pairs without
any special expression effect optionally being present
at both ends.

The isolated, double-stranded deoxyribonucleic acids
10 according to the invention contain the individual sub-
sequences in an operable sequence, i.e. they are in
functional relationship to one another. This means that
a promotor is present which has the recognition sequence
for the RNA-polymerase and that it is followed by a binding
15 site for the ribosomes. Then follows a start codon operable
in bacillus, preferably the start triplet GTG. The start
triplet is followed by the actual signal sequence for the
leader protein and then for the matured subtilisin Carlsberg
or its variants or sub-units. The leader sequence is
20 understood to be a prosequence, a presequence or a pre-/pro-
sequence which is preferably responsible for the excretion
of the enzyme from the cell. Finally, the structural
sequence is terminated by a stop codon, preferably by a
stop triplet recognized by Bacillus, such as for example
25 TAA. This may be followed by a terminator sequence,
preferred sequences being those which are capable of pairing
in themselves and which thus lead to loop formation
(stem loop). The function of terminator sequences such
as these is to terminate the synthesis of the messenger-RNA
30 for the particular gene product.

The isolated, double-stranded deoxyribonucleic acids
described may be adjoined at both ends by further sequences,
preferably those to which no special expression effect
can be ascribed.

35 The sequence for the matured enzyme may be subject



- 1 to certain variations within the scope of the invention.
In a first embodiment, sequences which code for subtilisin Carlsberg are provided. Preferred sequences are sequences made up solely of codons which are usual in *Bacillus*.
- 5 In one preferred embodiment of the invention, isolated deoxyribonucleic acids containing the following sequence for the matured enzyme are claimed:

	10	20	30	40
10	...GCGCAAA	CCGTTCTTA	CGGCATTCT	CTCATTAAG
	CGGACAAAGT	GCAGGCTCAA	GGCTTTAAGG	GAGCGAATGT
	AAAAGTAGCC	GTCTGGATA	CAGGAATCCA	AGCTTCTCAT
	CCGGACTTGA	ACGTAGTCGG	CGGAGGAAGC	TTTGTGGCTC
	GCGAAGCTTA	TAACACCGAC	GGCAACGGAC	ACGGCACACA
15	TGTTGCCGGT	ACAGTAGCTG	CGCTTGACAA	TACAACGGGT
	GTATTAGGCG	TTGCGCCAAG	CGTATCCTTG	TACGCGGTTA
	AAGTACTGAA	TTCAAGCGGA	AGCGGATCAT	ACAGCGGCAT
	TGTAAGCGGA	ATCGAGTGGG	CGACAACAAA	CGGCATGGAT
	GTTATCAATA	TGAGCCTTGG	GGGAGCATCA	GGCTCGACAG
20	CGATGAAACA	GGCAGTCGAC	AATGCATATG	CAAGAGGGGT
	TGTCGTTGTA	GCTGCAGCAG	GGAAACGCGG	ATCTTCAGGA
	AACACGAATA	CAATTGGCTA	TCCTGCGAAA	TACGATTCTG
	TCATCGCTGT	TGGTGGCGTA	GACTCTAACA	GCAACAGAGC
	TTCATTTTCC	AGCGTCGGAG	CAGAGCTTGA	AGTCATGGCT
25	CCTGGCGCAG	GCCTATACAG	CACCTACCCA	ACGAACACTT
	ATGCAACATT	GAACGGAACG	TCAATGGCTT	CTCCTCATGT
	AGCGGGAGCA	GCAGCTTTGA	TCTTGTCAAA	ACATCCGAAC
	CTTTCAGCTT	CACAAGTCCG	CAACCGTCTC	TCCAGCACGG
	CGACTTATTT	GGGAAGCTAA	TTCTACTATG	GGAAAGGTCT
30			stop	
	GATCAATGTC	GAAGCTGCCG	CTCAA	TAA...

This sequence codes for a ^{matured} subtilisin Carlsberg which deviates in positions 158 and 161 in the amino acid sequence.

In another embodiment of the invention, the claimed



isolated, double-stranded deoxyribonucleic acids code for non-modified subtilisin Carlsberg, i.e. they contain the amino acid asparagine instead of the amino acid serine in position 161 and the amino acid serine instead of the amino acid asparagine in position 158. It follows from this that

5 the base triplets (genetic codes) usual in *Bacillus* for these amino acids must be present in the corresponding isolated deoxyribonucleic acids.

The isolated, double-stranded deoxyribonucleic acids contain a sequence for a leader protein between the start triplet and the sequence coding for the matured enzyme.

10 The present invention also relates to hybrid plasmids for microorganisms of the genus *Bacillus*, characterized in that they contain at least one double-stranded DNA of the invention in operable form. According to the invention, a hybrid plasmid such as this is intended to have the following structure. It should have one resistance marker at its disposal

15 and should comprise at least one enzymatic cleavage site for specific restriction enzymes, as few cleavage sites as possible, i.e. no more than 4 and preferably no more than 1 cleavage site, being present per restriction enzyme. In other words the hybrid plasmids are intended to be able to be linearized by the use of special enzymes.

20 The invention further provides a process for selecting microorganism strains containing hybrid plasmids of the invention, characterized in that

- In a first selection process, the strains are separated from strains free from hybrid plasmid through the property of resistance acquired by the hybrid plasmid and

25 - the producers of subtilisin Carlsberg are selected from the resistant strains by coating the plate cultures with a film covered with antibodies against subtilisin Carlsberg and binding the antibody-subtilisin Carlsberg complex to other antibodies against subtilisin Carlsberg which



are coupled to a peroxidase, after which a color reaction is carried out with the peroxidase.

The invention also provides microorganism strains of the genus *Bacillus* containing hybrid plasmids of the invention.

- 5 The invention provides a process for the preparation of subtilisin Carlsberg, characterized in that microorganism strains of the invention are cultivated.

10 The function of the resistance marker is to enable microorganisms containing the hybrid plasmids, which have been transformed in this way, to be distinguished by their resistance from those which do not contain these hybrid plasmids. Various starting plasmids are available to the expert in this field. They have resistance markers against, for example, tetracycline, kanamycin, chloramphenicol or other antibiotics. Among the various possible starting plasmids for preparing the hybrid plasmids, those
15 which are suitable for microorganisms of the genus *Bacillus* should



- 1 be selected. Accordingly, the hybrid plasmids according
to the invention are best obtained from starting plasmids
which in turn are suitable for bacillus. Various starting
plasmids of this type have been described in the specialist
5 literature. A large number of suitable plasmids can be
ordered from depositories, for example from the Bacillus
Genetic Stock Center. Suitable starting plasmids are,
for example the following: pBCE16, pC194, pUB110, pE194,
pSA2100, pPL608, pBD64. References to these plasmids can
10 be found in the following literature:

Bernhard K., Schrempf H., Goebel W.,
1978, J. Bacteriol. 133: 897 - 903

- 15 Gryczan, T.J., Contente, S., and Dubnau, D.
1978, J. Bacteriol. 134: 318 - 329

Williams, D.M., Duvall, E.J. and Lovett, P.S.,
1981, J. Bacteriol. 146: 1162 - 1165

- 20 Gryczan, T., Shivakumar, A.G., and Dubnau, D.,
1980, J. Bacteriol. 141: 246 - 253

- 25 Ehlich, S.D., Bursztyn-Pettegrew, H., Stroynowski, J., and
Leaderberg, J., 1977 in Recombinant Molecules: Impact on
Science and Society, pp. 69 - 80, Raven Press, New York

Weisblum, B. Graham, M.Y., Gryczan, T., and Dubnau, D.
1979, J. Bacteriol. 137: 639 - 643

- 30
When selecting suitable starting plasmids, it is
important to remember that they should be able to be
linearized by specific restriction enzymes without losing
their characteristic properties. The characteristic
35 properties of the starting plasmids are understood to include

- 1 their replicatability, their resistance and the possibility
for expression in *Bacillus*. Current specific restriction
enzymes with which suitable starting plasmids can be
linearized are, for example, Bam HI, Eco RI, H_{in} DIII and
5 Pst I.

The hybrid plasmids according to the invention contain
the double-stranded isolated deoxyribonucleic acids inserted
in one or more such cleavage sites. For example, the
starting plasmid pBCE16 which is particularly suitable
10 for the purposes of the invention may be linearized with
Bam HI and then reclosed to form the ring after insertion
of the double-stranded deoxyribonucleic acids according
to the invention. Another embodiment of the invention
is directed not only to the ring-closed hybrid plasmids,
but also to the corresponding ring-opened forms and also
15 adducts of 2, 3 or more ring-opened hybrid plasmids.

The present invention also relates to microorganism
strains of the genus *Bacillus* which contain hybrid plasmids
of the type claimed in Claim 2 and the following Claims.
20 For producing or rather transforming suitable microorganism
strains, the expert will choose those strains which
accommodate hybrid plasmids and in which these hybrid plasmids
show adequate stability. Strains of the species *Bacillus*
subtilis, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*
25 or even *Bacillus cereus* may advantageously be transformed.
In this connection, transformed strains may be distinguished
from non-transformed strains by detections of the protease
formed, for example by the immunological method described
hereinafter.

- 30 In one embodiment of the invention, the strains trans-
formed are *Bacillus* strains, for example *Bacillus subtilis*
strains, which for their part are not able to form protease.
Strains such as these may be obtained from depositories.
It is thus possible to obtain strains which are only capable
35 of producing the desired subtilisin Carlsberg or the



- 1 described variant of subtilisin Carlsberg. However, it
is also possible similarly to produce strains which thus
secrete subtilisin Carlsberg in addition to other proteases
or amylases. Specifically, it is thus possible by selection
5 to obtain strains showing particularly high enzyme production.

The double-stranded deoxyribonucleic acids according
to the invention are isolated from protease-forming strains
of the species *Bacillus licheniformis*, among which the
following strains for example are suitable: *Bacillus*
licheniformis: ATCC 10716, DSM 641 as well as the strains
DSM 3406 and DSM 3407 (Laboratory specification "A 441 and
"A 453" (sent to DSM for deposition by letter of

- 19.7.85). These strains are cultivated as described in
German Patent 29 25 427. The cells are then harvested.
The harvested cells are subjected to protoplasia, i.e.
15 themurein cell wall is disrupted with suitable enzymes,
particularly lysozyme, after which the actual cell membrane
is disrupted with detergents, such as for example sodium
dodecylsulfate. The lysates thus formed may first be
subjected to a treatment with RNA-cleaving enzymes (RNase)
20 and/or proteinase K. Then follows the DNA isolation step.

- Several methods are known to the expert for isolating
chromosomal DNA. They may be carried out either individually
or in combination. In one important method, the lysate
is first subjected to fractional precipitation in ethanol
25 to enable high molecular weight DNA-fragments to be separated
off and thus isolated. Another method is based on separation
in a density gradient. To this end, a density gradient
is produced in a caesium chloride/DNA-solution. The total
quantity is divided up into various fractions so that the
30 DNA collects in certain of these fractions which may then
be separated off and further processed. In many cases,
it is best to eliminate most of the proteins before or
during isolation of the DNA by extraction with a mixture
of chloroform and isoamylalcohol.

- 35 Basically, the plasmid-DNA may be separated off in

1 the same way, although in this case it is best to introduce
ethidium bromide (a red dye capable of intercalation) into
the caesium chloride density gradient, thus artificially
establishing a difference in density between plasmid and
5 chromosomal-DNA.

The purified, isolated deoxyribonucleic acids are
then subjected to cleavage with restriction enzymes in
a suitable buffer (at pH values of from 7 to 7.5). Suitable
restriction enzymes are, for example, Bam HI, Sau 3A or
10 other enzymes which are capable of cleaving DNA at specific
sites. The chromosomal DNA may be treated with the same
enzymes as the plasmid DNA or even with different enzymes.
In either case, it is important to ensure that cleavage
of the chromosomal DNA is not complete in order thus to
15 obtain fragments of adequate length which with sufficient
probability contain the structural gene intact. The
isolated, double-stranded deoxyribonucleic acids are mixed
with the linear plasmids, the chromosomal deoxyribonucleic
acids preferably being used in a large excess by weight
20 over the linearized plasmids. A ratio of from 1:2 to
1:20 is preferably used, a ratio of from 1:5 to 1:10 being
particularly preferred. The mixed deoxyribonucleic acids,
which should then be present in a concentration of around
0.2 mg per ml, are ligated with DNA-ligase in a following
25 step.

The ligation mixture thus obtained is used to transform
competent cells of *Bacillus* species, such as *Bacillus subtilis*
or *Bacillus licheniformis*. If it is desired to recognize
successfully transformed strains from their protease formation,
30 it is preferred to transform those strains which, for their
part, are not capable of forming protease or at least are
not capable of forming subtilisin Carlsberg. However,
the starting strains may also be transformed. In order
to obtain competent cells, the strain intended for trans-
35 formation is cultivated to the stationary phase in a first



1. minimal medium and is then further cultivated in a second, dilute minimal medium. The hybrid plasmid is then offered as such to the microorganisms thus pretreated, generally in buffer solution. Thereafter a growth phase may be
- 5 carried out in a full medium (literature Cahn, F.H. and Fox, M.S. (1968) J. Bacteriol. 95: 867 - 875). The microorganisms thus obtained are then subjected to a selection process. In this process, they are normally first cultivated in a medium containing an antibiotic against which the
- 10 hybrid plasmid imparts resistance. In this way, organisms actually containing the hybrid plasmid in one or more copies may be obtained in a first selection step. The microorganisms are then selected for their ability to form subtilisin Carlsberg in another selection step.

- 15 An immunological method may advantageously be used for this selection step. A preferred method is based on the work of Broome and Gilbert. In this method, antibodies against subtilisin Carlsberg are first obtained by immunization of rabbits, as described by Buckel. Some
- 20 of the antibodies are enriched by precipitation with ammonium sulfate and DEAE-chromatography whilst the rest are additionally enriched by affinity chromatography with activated Sepharose 4B.

- 25 Proof of the existence of subtilisin Carlsberg may be provided as follows:

- A PVC film is coated with antibodies against subtilisin Carlsberg. The coated film is placed on the bacterial cultures to be investigated. Any subtilisin Carlsberg present is bound by the antibodies. In another step,
- 30 the subtilisin Carlsberg antibodies purified by affinity chromatography, onto which peroxidase has been coupled in a previous step, are bound to the antibody-subtilisin Carlsberg complex.

- 35 (1) Broome, S. & Gilbert, W. (1978) Proc. Natl. Acad. Sci. USA 75, 2746 - 2749

- 1 (2) Buckel, P & Zehelein, E. (1981) Gene 16, 149 - 159

The detection is carried out with tetramethylbenzidine as the peroxidase substrate. In the presence of subtilisin Carlsberg-positive clones, a blue-green coloration develops.

The immunological separation method described above affords considerable advantages in connection with the present invention. Thus, it enables strains which actually form subtilisin Carlsberg to be specifically recognized.

- 10 This is particularly important in cases where the strains used also comprise other metabolism products which do not respond to the immunological method. Another advantage of this method of detection is that it enables the clones to be safely isolated from a large number of transformed
15 and non-transformed strains.

From the clones thus isolated which contain the hybrid plasmids according to the invention, the hybrid plasmids may be isolated as described at the beginning by cell lysis and subsequent separation in the density
20 gradient in the presence of ethidium bromide. Purified hybrid plasmids are made available in this way. Trouble-some ethidium bromide may be separated off by extraction with isopropanol. The purified hybrid plasmids may be inserted into other *Bacillus* strains under the conditions
25 described above.

- In another embodiment of the invention, the transformation may be carried out using protoplasts rather than competent cells. To this end, protoplasts are initially prepared by disrupting the murein cell wall of microorganisms
30 of the genus *Bacillus* with suitable enzymes and then allowing the hybrid plasmids to act together with polyethylene glycol on the protoplasts, preferably working in buffer solutions. The protoplasts are then further cultivated on a regeneration medium and the intact micro-organisms thus obtained, which contain the hybrid plasmid
35



- 1 according to the invention, are separated off from other strains by the selection processes mentioned above.

EXAMPLES

5

Chromosomal DNA from *Bacillus licheniformis* DSM 641 or even ATCC 10716 was isolated by Marmur's method (1) with the following modifications: the cells were incubated for 20 minutes in ice in 1 mg lysozyme per ml in 10 mM tris-hydrochloride pH 7.5 containing 25% saccharose before EDTA and sodium dodecylsulfate were added. The extraction with chloroform-isoamylalcohol was carried out only once.

Using a vertical rotor, the DNA was centrifuged for 20 hours at 42,000 r.p.m./20°C in a density gradient (density 1.71 g/cm³), separated off from other cell constituents and fractionated by dripping out.

Plasmid-DNA from *Bacillus subtilis* was isolated by Bernhard's method (1), the plasmid-DNA being drawn off by means of a cannula after the centrifuging step in a density gradient.

pBCE 16 (2,4) was used as the vector plasmid for cloning in *Bacillus subtilis* BR 151 (3).

In order to obtain the gene for subtilisin Carlsberg intact, partial cleavage of the chromosomal DNA of *Bacillus licheniformis* DSM 641 or ATCC 10716 was carried out with the restriction endonuclease *Sau* 3A.

Since *Sau* 3A recognizes a 4-sequence, whereas *Bam* HI recognizes a 6-sequence, i.e. *Sau* 3A cuts more frequently, the cleavage sites for incomplete cleavage with *Sau* 3A are more uniformly distributed than for cleavage of the chromosomal DNA with *Bam* HI.

Cleavage was carried out, as in all the following cases, under the conditions of the Maniatis (5) manual, except that for the incomplete cleavage the incubation time which would normally be 1 hour was correspondingly

- 1 shortened in order to obtain cleavage products comparable
in their center of gravity with the vector pBCE 16 (approx.
3 Md). Approx. 1 unit of enzyme was used per 1 μ g DNA.
The vector pBCE 16 was linearized with the restriction
5 endonuclease Bam HI which allows the same overlapping ends
to be formed as Sau 3A.

After incubation, the enzyme was extracted twice
with half the volume of phenol, after which Bam HI-cleaved
pBCE 16 and Sau 3A-cleaved chromosomal DNA were mixed in
10 a quantitative ratio of 1:10, the mixture was extracted 5 times
with the same volume of ether and then incubated for 20
minutes at -70°C with twice the volume of ethanol and the
DNA pelleted by centrifuging.

After drying, the DNA was dissolved in the following
15 buffer: 66 mM tris-(hydroxymethyl)-aminomethane, 5 mM MgCl_2 ,
0.3 mM adenosine triphosphate, 1.5 mM dithiothreitol and
0.07 mg/ml beef serum albumin.

The DNA concentration was adjusted to 200 μ g/ml and
T4-DNA-ligase was added in a concentration of 1 U per μ g
20 DNA used. The ligation reaction was carried out for approx.
18 hours at 16°C . The ligation mixture, i.e. the DNA-
fragments ligated with T4-DNA-ligase, were then used for
the transformation of competent cells of *Bacillus subtilis*
BR 151.

25 Competent cells of *B. subtilis* BR 151 were prepared
by Laird's method, as described by Cahn and Fox (6).

0.18 ml 0.1 M MgCl_2 , 0.13 ml 0.05 M CaCl_2 and 0.13 ml
100 mM EGTA (ethyleneglycol-bis-2-aminoethylether-N,N',N'-
tetraacetate) pH 7.3 were added to and mixed (in this order)
30 with 0.82 ml of competent cells, followed after gentle
shaking for 5 minutes at 30°C by the addition of 10 μ l
or 10 μ g of the ligation mixture. After slow shaking
(60 r.p.m.) for 30 minutes at 30°C , 1 ml of 2 x HGP (10 g
peptone from casein, 5 g yeast extract, 5 g NaCl and 5 g
35 glucose/per litre was added.

- 1 After shaking for 90 minutes at 160 r.p.m./30°C, the
cells were spread with a glass spatula onto calcium-caseinate-
agar (modified after Frazier and Rupp - Merck) additionally
containing 0.5% casein and 15 µg/ml tetracycline for the
5 purpose of selection of the transformed cells (100 µl per
plate).

The plates were incubated for 48 hours at 37°C.

- Using subtilisin Carlsberg-specific antibodies purified
by chromatography on diethylaminoethylcellulose (DEAE)
10 in combination with a Boehringer/Mannheim "test kit for
the immunological detection of specific gene expression
in microorganisms", it was possible to detect 6 protease-
positive clones among a total of approximately 32,000
transformants. After repeated inoculation, one of these
15 clones proved to be sufficiently stable for further
investigations. The detection of s. Carlsberg was carried
out as follows:

- PVC films were washed in isopropanol for 2 minutes
to remove grease and then dried. 15 films were then
20 incubated for 10 minutes in 40 ml 0.2 M sodium carbonate
buffer pH 9.2 containing 0.6 ml DEAE-purified subtilisin
Carlsberg-specific IgG-fraction for the purpose of coating.
The films were transferred without drying to an after-
coating bath and left therein for 10 minutes:

- 25 40 ml PBS-buffer*
+ 4.8 mg beef globulin
+ 40 mg beef serum albumin
*) PBS buffer pH 7.5: 0.05 M KH_2PO_4 ; 0.1 M NaCl
30

The films were then dried between paper towels.

- The IgG-coated films were then placed on the covered
agar plates and incubated for 2 hours at room temperature.
After removal of the films, the adhering cells were rinsed
35 off with cold tapwater, after which the films were rinsed

- 1 on both sides with PBS-buffer tempered to $60^{\circ}\text{C} + 0.1\%$ beef serum albumin. Without drying, the films were transferred to 50 ml of conjugate bath (50 ml PBS buffer containing 0.1% beef serum albumin + 1 μl peroxidase-conjugated IgG
- 5 against subtilisin Carlsberg) and left therein for 4 hours at room temperature.

The films were then rinsed with cold tapwater and then on both sides with PBS-buffer tempered to $6^{\circ}\text{C} + 0.05\%$ Tween 20 + 0.1% beef serum albumin. After drying between

10 paper towels, the films were incubated on a substrate (Boehringer Gen-Expressionskit^(R), Boehringer Mannheim Gm 611) for the coupled peroxidase. The substrate in question is tetramethylbenzidine dissolved in a gelatin base. After incubation for 10 to 30 minutes at room

15 temperature, a blue-green coloration develops in the presence of positive colonies, i.e. in the presence of subtilisin Carlsberg.

The plasmid-DNA of the stable s. Carlsberg-positive clone (pC 50) was isolated as described above.

For characterization, the pC 50-DNA was subjected to cleavage with various restriction enzymes both individually and in combination with Ava I, Bam HI, Bal I, Eco RI, Hpa II, Pst I and Sst I.

20

Analysis of the cleavage products separated up by

25 vertical agarose gel electrophoresis (7) produced the plasmid map in the accompanying drawing.

- 1 Both the Eco RI/Pst I-fragments concerning the cloned
fragment and the Eco RI-fragment joining up on the left
were inserted into the sequencing vectors pEMBL 8 and
pEMBL 9 (8) and the sequence determined by the chain
5 termination dideoxy method (9).

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The claims defining the invention are as follows:

1. Isolated, double-stranded deoxyribonucleic acids consisting of
- the following structural gene which only codes for a polypeptide:

10	20	30	40
...GCGCAAA	CGGTTCCTTA	CGGCATTCCCT	CTCATTAAAG
CGGACAAAGT	GCAGGCTCAA	GGCTTTAAGG	GAGCGAATGT
AAAAGTAGCC	GTCCGTGGATA	CAGGAATCCA	AGCTTCTCAT
CCGGACTTGA	ACGTAGTCGG	CGGAGGAAGC	TTTGTGGCTC
GCGAAGCTTA	TAACACCGAC	GGCAACGGAC	ACGGCACACA
TGTTGCCGGT	ACAGTAGCTG	CGCTTGACAA	TACAACGGGT
GTATTAGGCG	TTGCGCCAAG	CGTATCCTTG	TACGCGGTTA
AAGTACTGAA	TTCAAGCGGA	AGCGGATCAT	ACAGCGGCAT
TGTAAGCGGA	ATCGAGTGGG	CGACACAAA	CGGCATGGAT
GTTATCAATA	TGAGCCTTGG	GGGAGCATCA	GGCTCGACAC
CGATGAAACA	GGCAGTCGAC	AATGCATATG	CAAGAGGGGT
TGTCGTTGTA	GCTGCAGCAG	GGAACAGCGG	ATCTTCAGGA
AACACGAATA	CAATTGGCTA	TCCTGCGAAA	TACGATTCTG
TCATCGCTGT	TGGTGCGGTA	GACTCTAACA	GCAACAGAGC
TTCAATTTCC	AGCGTCGGAG	CAGAGCTTGA	AGTCATGGGT
CCTGGCGCAG	CGGTATACAG	CACTTACCCA	ACGAACACTT
ATGCAACATT	GAACGGAACG	TCAATGGGTT	CTCCTCATGT
AGCGGGAGCA	GCAGCTTTGA	TCTTGTCAAA	ACATCCGAAC
CTTTCAGCTT	CACAAGTCCG	CAACCGTCTC	TCCAGCACGG
CGACTTATTT	GGGAAGCTAA	TTCTACTATG	GGAAAGGTCT
		stop	
GATCAATGTC	GAAGCTGCCG	CTCAA	TAA....

- start and stop codons in an operable sequence,
- a ribosome binding site,
- a promoter,

characterized in that it has the following structure:



- 20A -

- a promoter which is recognized by the RNA-polymerase of microorganisms of the genus *Bacillus*,

- a ribosome binding site,
- a start codon,
- a leader sequence,
- a stop codon,

DNA-sequences containing up to 400 base pairs without any special expression effect optionally being present at both ends.

2. Hybrid plasmids for microorganisms of the genus *Bacillus*, characterized in that they contain at least one double-stranded DNA according to Claim 1 in operable form.

3. Hybrid plasmids as claimed in Claim 2, characterized in that they contain a resistance marker and, for the insertion of foreign DNA, at least one enzyme-specific cleavage site, preferably no more than 1 cleavage site being present per restriction enzyme.

4. Hybrid plasmids as claimed in Claim 2 or 3, characterized in that they contain a resistance marker and, for the insertion of foreign DNA, cleavage sites for specific DNA-cleaving enzymes, preferably no more than four and more preferably only one cleavage site being present for each restriction enzyme.

5. Hybrid plasmids as claimed in any one of Claims 2 to 4,



characterized in that they are derived from pBCE 16 and the deoxyribo-nucleic acids according to Claim 1 contain Bam HI inserted into the cleavage site.

6. A process for selecting microorganism strains containing hybrid plasmids according to Claims 2 to 5, characterized in that

- in a first selection process, the strains are separated from strains free from hybrid plasmid through the property of resistance acquired by the hybrid plasmid and

- the producers of subtilisin Carlsberg are selected from the resistant strains by coating the plate cultures with a film covered with antibodies against subtilisin Carlsberg and binding the antibody-subtilisin Carlsberg complex to other antibodies against subtilisin Carlsberg which are coupled to a peroxidase, after which a color reaction is carried out with the peroxidase.

7. Microorganism strains of the genus bacillus containing hybrid plasmids according to Claims 1 to 6.

8. Microorganism strains as claimed in Claim 7, characterized in that they belong to the species *Bacillus subtilis* or *Bacillus licheniformis*.

9. A process for the preparation of subtilisin Carlsberg, characterized in that microorganism strains according to Claims 7 and 8 are cultivated.

10. Isolated, double-stranded deoxyribonucleic acids substantially as hereinbefore described with reference to any one of the Examples.

11. Isolated, double-stranded deoxyribonucleic acids substantially as hereinbefore described with reference to the accompanying drawings.

12. Hybrid plasmids substantially as hereinbefore described with reference to any one of the Examples.

13. Hybrid plasmids substantially as hereinbefore described with reference to the accompanying drawings.

14. A process for selecting microorganism strains containing hybrid plasmids substantially as hereinbefore described with reference to any one of the Examples.

15. Microorganism strains of the genus bacillus containing hybrid plasmids substantially as hereinbefore described with reference to any one of the Examples.



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16. A process for the preparation of subtilisin Carlsberg substantially as hereinbefore described with reference to any one of the Examples.

DATED this SIXTEENTH day of OCTOBER 1989
Henkel Kommanditgesellschaft Auf Aktien

Patent Attorneys for the Applicant
SPRUSON & FERGUSON



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